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Comparison in the immunological properties of *Borrelia burgdorferi* isolates from *Ixodes ricinus* derived from three endemic areas in Switzerland

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SUMMARY

Borrelia burgdorferi isolates were obtained from *Ixodes ricinus* from three sites in Switzerland. They were examined by SDS–PAGE and immunoblotting. The phenotypes, in respect of three outer surface proteins (Osp), differed between the sites of collection. In site 1, most isolates had an OspA of 31 kDa and an OspB of 34 kDa; in site 2, isolates presenting an OspA of 33 kDa dominated and in site 3, the isolates with an OspA of 32 kDa and an OspB of 35 kDa were most frequent. This distribution differed significantly. About half of the isolates from sites 1 and 3 reacted with anti-OspA monoclonal antibody H5332 compared to 29% from site 2. Site 1 isolates reacted significantly more frequently (81%) with another anti-OspA monoclonal antibody LA-31 than isolates from site 3 ($P < 0{\cdot}0001$). These findings have implications for the epidemiology of Lyme borreliosis, for the further development of serodiagnostic reagents and for the development of a vaccine.

INTRODUCTION

In humans, Lyme borreliosis is a disease caused by infection with *Borrelia burgdorferi* [1]. *B. burgdorferi* is transmitted by infected ticks belonging primarily to the *Ixodes ricinus* complex [2]. In Europe, *B. burgdorferi* can be isolated from infected ticks, animals and patients. The outer membrane of *B. burgdorferi* contains at least three outer surface proteins (Osp) A (31–33 kDa), B (34–36 kDa) and C (20–24 kDa) [3–6]. These lipoproteins are embedded in the fluid outer membrane of *B. burgdorferi* and are encoded by linear plasmids [6, 7]. Although their exact functions have not yet been defined, the outer surface proteins of *B. burgdorferi* are generally thought to have an important role in the host–parasite interactions during the course of infection. The European isolates are more heterogeneous with respect to their antigenic profiles than the American isolates [8–12].

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Table 1. *Characterization of* B. burgdorferi *isolates* (n = *92*)

	Osp Mws (kDa)			MoAbs								PoAb
Strains	A	B	C	H5332	LA-2	LA-4	LA-31	LA-25	LA-27	LA-7	p39	anti-22 kDa
Site 1												
NE83	33	a	22	−	−	−	+	−	−	−	+	+
NE84	33	a	22	−	−	−	+	−	−	−	−	+
NE85	33	a	22	−	−	−	+	−	−	−	−	+
NE190	31	34	23	−	−	−	+	−	+	−	−	+
NE192	33	a	22	−	−	−	−	−	−	−	−	−
NE193	31	34	a	−	−	−	+	−	+	−	−	−
NE308	33	34	a	−	−	−	+	−	−	−	−	+
NE317	32	34	22	−	−	−	+	−	−	−	−	+
NE323	32	35	a	−	−	−	−	−	−	−	−	+
NE15	31	34	a	+	−	−	+	−	+	−	−	+
NE12	31	34	22	+	+	+	+	+	+	+	+	−
NE14	31	34	a	+	+	−	−	−	−	−	+	−
NE196	31	34	a	+	+	−	+	−	+	−	+	−
NE303	32	35	23	−	+	−	+	−	+	−	−	−
NE304	32	a	22	−	−	−	−	−	−	−	−	−
NE305	31	34	22	−	+	+	+	+	+	−	−	−
NE378	33	a	22	−	−	−	+	−	+	−	−	−
NE20	32	a	23	+	−	−	+	−	−	−	n	−
NE25	31	34	23	+	+	−	+	+	+	+	n	+
NE26	33	a	22	+	−	−	+	−	−	−	n	−
NE8	32	a	23	+	−	−	+	−	−	−	n	−
NE19	32	35	a	+	−	−	−	−	−	−	n	−
NE550	31	34	a	+	+	−	+	+	+	+	n	−
NE21	32	a	22	+	+	−	+	−	−	−	n	−
NE24	31	34	23	+	+	+	+	+	+	+	n	+
NE23	32	a	22	+	+	−	+	−	−	−	n	+
Site 2												
NE2	33	a	22	−	−	−	+	−	−	−	−	−
NE4	33	a	22	−	−	−	−	−	−	−	−	+
NE58	33	a	a	−	+	−	+	−	−	−	+	−
NE60	33	a	a	−	+	−	+	−	−	−	+	−
NE173	33	a	22	−	−	−	−	+	−	−	−	+
NE352	32	34	23	+	+	−	−	−	+	−	−	+
NE355	33	a	22	−	−	−	+	−	−	−	−	+
NE363	32	35	a	−	−	−	−	−	−	−	−	−
NE3	33	a	a	−	−	−	n	n	n	−	n	n
NE9	32	a	22	+	−	−	+	−	−	−	n	+
NE10	32	a	23	+	−	−	+	−	−	−	n	+
NE22	33	a	a	−	−	−	+	−	−	−	n	−
NE16	32	a	22	+	−	−	−	−	−	−	n	−
NE1	32	a	22	−	−	−	−	−	−	−	n	+
NE5	33	a	22	−	−	−	−	−	−	−	n	+
NE6	33	a	a	−	−	−	−	+	−	−	n	−
NE27	32	35	23	+	−	−	−	−	−	−	n	−
Site 3												
NE413	33·5	a	22	−	−	−	−	−	−	−	−	+
NE443	31	a	23	+	−	+	−	−	−	−	−	+
NE450	32	35	23	+	−	−	−	−	−	−	+	+
NE454	32	35	21	−	−	−	−	−	−	−	−	+
NE456	31	34	22	+	+	−	+	+	+	+	+	+
NE460	32	35	22	+	+	−	−	−	−	−	−	−

Table 1 (*cont.*)

	Osp Mws (kDa)			MoAbs								PoAb anti-
Strains	A	B	C	H5332	LA-2	LA-4	LA-31	LA-25	LA-27	LA-7	p39	22 kDa
NE461	32	35	22	−	−	−	−	−	−	−	−	+
NE462	33	a	23	+	−	+	−	−	−	−	−	+
NE472	32	35	23	+	−	−	−	−	−	−	+	+
NE474	31	a	23	−	−	+	−	−	−	−	−	+
NE477	32	35	22	+	−	−	−	−	−	−	+	+
NE478	32	35	22	−	−	−	−	−	−	−	−	+
NE485	32	35	22	+	−	−	−	−	−	−	+	+
NE490	32	a	22	+	−	−	+	−	−	−	−	+
NE496	32	35	23	+	−	−	−	−	−	−	+	−
NE506	a	a	22	−	−	−	−	−	−	−	−	+
NE507	32	35	22	−	−	−	−	−	−	−	+	+
NE517	32	35	22	−	−	−	−	−	−	−	−	+
NE519	32	35	22	−	−	−	−	−	−	−	−	+
NE558	32	a	22	+	−	−	+	−	−	−	−	+
NE601	32	a	21	−	−	−	+	−	−	−	−	+
NE603	32	a	22	+	−	+	−	−	−	−	−	+
NE606	32	35	22	+	−	−	−	−	−	−	−	+
NE607	32	a	22	+	−	−	+	−	−	−	+	+
NE608	32	a	22	+	−	−	+	−	−	−	−	+
NE623	32	35	22	+	−	−	−	−	−	−	−	+
NE624	32	35	22	+	−	−	−	−	−	−	+	+
NE629	32	35	a	+	−	−	−	−	−	−	+	+
NE630	32	35	22	+	−	−	−	−	−	−	+	+
NE632	32	35	23	+	−	−	−	−	−	−	+	+
NE418	32	35	a	+	+	−	+	−	−	−	−	−
NE426	33	a	22	+	+	−	−	−	−	−	−	+
NE429	32	35	a	−	+	−	−	−	−	−	−	−
NE435	32	35	22	−	−	−	−	−	−	−	−	−
NE438	33	a	a	−	+	−	−	−	−	−	−	−
NE463	31	34	21	+	+	−	+	+	+	+	+	+
NE467	32	35	21	+	+	−	−	−	−	−	−	+
NE471	32	35	22	−	+	+	−	−	−	−	−	+
NE470	32	35	a	−	+	−	−	−	−	−	−	−
NE493	31	34	21	+	−	+	+	+	+	+	+	+
NE508	32	35	22	+	+	−	+	−	−	−	−	+
NE537	32	35	22	−	+	−	−	−	−	−	−	−
NE605	32	35	22	−	+	−	−	−	−	−	−	+
NE200	32	a	22	+	+	−	+	+	+	−	−	+
NE207	32	a	23	+	+	+	−	+	−	−	−	+
NE201	32	a	a	−	+	−	+	−	−	−	+	−
NE202	31	a	22	−	+	+	+	−	−	−	−	−
NE203	32	35	a	+	+	+	+	+	+	−	+	−
NE204	32	36	a	−	−	−	−	−	−	−	−	−

+, positive reaction; −, negative reaction; a, absent; n, not tested.

In this study, *B. burgdorferi* strains from *B. burgdorferi* ticks collected in three different endemic areas were screened for evidence of phenotypic differences using immunochemical methods.

MATERIAL AND METHODS

Collection of ticks and isolation of B. burgdorferi

I. ricinus ticks (adults and nymphs) were collected by flagging lower vegetation using a white cotton flannel flag (1 m^2) which was dragged behind collectors in three endemic areas: site 1, Bois de l'Hôpital forest close to Neuchâtel; site 2, Staatswald forest and site 3, Karoline forest around Aarberg. Ticks were collected during April–June and August–November 1987 to 1992 in sites 1 and 2, and in 1989 and 1992 in site 3. For isolation of *B. burgdorferi*, the midgut of the tick was incubated for 10 days at 34 °C in individual culture tubes containing 4 ml BSK II medium [13] supplemented with rifampicin (50 μg/ml) and phosphomycin (50 μg/ml).

SDS–PAGE and immunoblot analysis

Each isolate was inoculated in 25 ml BSK II medium and after 10 days, the cultures were centrifuged and washed twice with PBS + 5 mM $MgCl_2$. Whole-cell lysates (equivalent to 10^7 cells/lane) were separated by SDS–PAGE using a 12·5% polyacrylamide gel. The gels were stained with Coomassie brilliant blue R 250 [14].

The separated proteins were transferred onto nitrocellulose paper using a transit cell (2117-250 Nova Blot Electrophoretic Transfer Kit, LKB AM Bromma, Sweden) [14]. The monoclonal antibodies (MoAbs): H5332, LA-2, LA-4, LA-31 (anti-OspA) [8, 15], LA-25, LA-27 (anti-OspB) [15], LA-7 (anti-20 kDa protein) [15], and polyclonal antibodies (PoAbs): anti-22 kDa/NE4 [14] and anti-B31, produced by immunizing a New Zealand white rabbit with strain B31 [9], were used for immuno-blotting. Bound antibodies were visualized by using peroxidase labelled anti-rabbit IgG or anti-mouse IgG antibodies (1:1000, Nordic Immunological Laboratories, The Netherlands).

Statistical analyses

The Fischer's exact test was used to compare the distribution of the different characterized *B. burgdorferi* isolates derived from different areas. The difference was considered as significant if P value was $< 0{\cdot}017$ [16].

RESULTS

Characterization of B. burgdorferi *isolates by immunological methods*

Twenty-six *B. burgdorferi* isolates were obtained from ticks of the Bois de l'Hôpital forest (site 1), 17 isolates from the Staatswald forest (site 2) and 49 isolates from the Karoline forest (site 3). OspA, OspB and OspC were expressed by 91 (99%), 50 (54%) and 70 (76%) of the 92 isolates (Table 1). Four different phenotypes could be distinguished on the basis of the expression pattern of the Osps, namely, A (11% of isolates), AB (16%), AC (41%) and ABC (32%).

Péter and colleagues [11] distinguished four typing groups of *B. burgdorferi* (I, II, III and IV) according to the molecular weight of OspA and OspB (Fig. 1*a*). Most of our isolates belong to groups I, II and III and only one, from site 3, is in group IV. Seven isolates did not fit into this classification and comprised three additional groups: V, VI and VII (Fig. 1*a*).

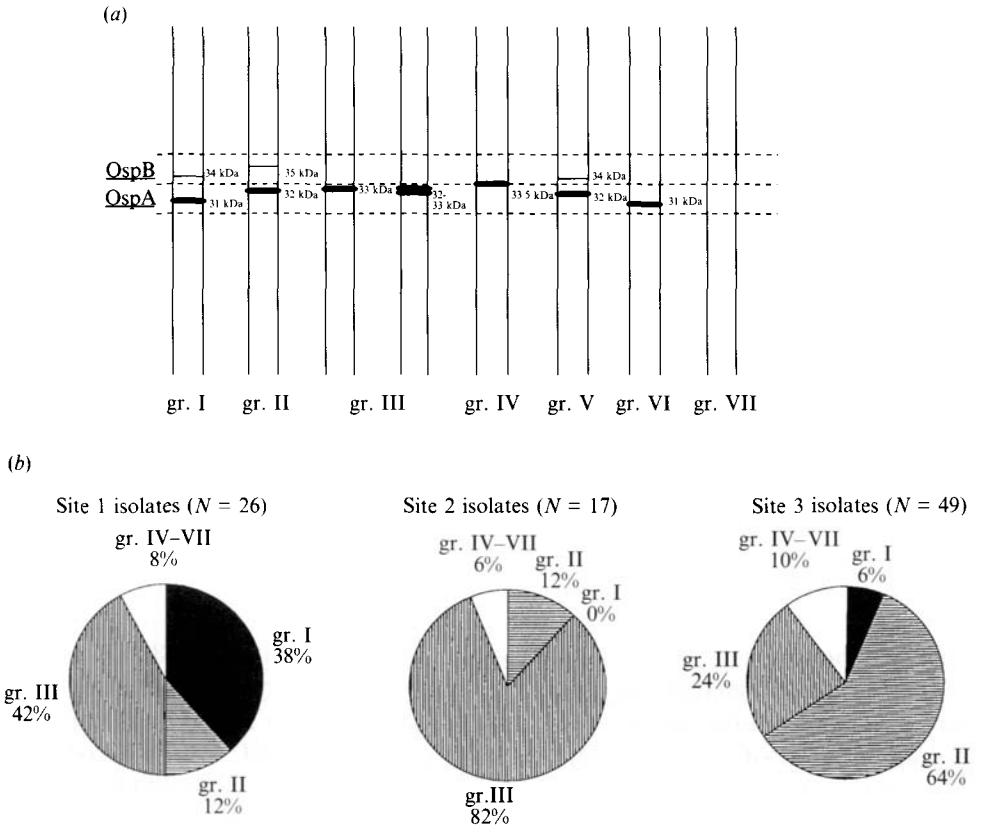

Fig. 1*a* and *b*. Typing groups of *B. burgdorferi* isolates and their geographical distribution. (*a*) Schematic description of the typing groups according to the molecular weights of OspA and OspB. The first four groups (gr. I–IV) were suggested by Péter and colleagues [11] and the groups V–VII were proposed by us. Group VII comprised one isolate (NE506) which did not express OspA and OspB. (*b*) Relative distribution of the different typing groups in the three sites of isolation: groups I and III prevailed in site 1, group III or II prevailed in site 2 and site 3, respectively.

Immunological characterization of these isolates revealed a heterogeneous reactivity with the different antibodies used (Table 1).

The different phenotypes obtained from April to November did not show any differences in their seasonal distribution (data not shown).

Distribution of the characterized isolates in the three studied sites

The isolates from the three different geographic sites of isolation were compared for: (1) the apparent molecular weights (Mw) of OspA, OspB and OspC; (2) their classification into the respective typing groups; (3) differences in the Osp phenotypes; (4) their reactivity with mono- and polyclonal antibodies specific for *B. burgdorferi* antigens.

The apparent molecular weights of OspA, OspB and OspC

The results are summarized in Table 2. OspA was present in nearly all isolates;

Table 2. *The presence of the major proteins in isolates derived from three different sites*

	OspA			OspB			OspC		
	31 kDa	32 kDa	33 kDa	34 kDa	35 kDa	36 kDa	21 kDa	22 kDa	23 kDa
Site 1	**10**	9	7	**12**	3	0	0	12	6
($N = 26$)	(38%)	(35%)	(27%)	(46%)	(12%)	(0%)	(0%)	(46%)	(23%)
Site 2	0	7	10	1	2	0	0	8	3
($N = 17$)	(0%)	(41%)	(59%)	(6%)	(12%)	(0%)	(0%)	(47%)	(18%)
Site 3	6	**38**	**3**	3	**29**	1	5	28	8
($N = 49$)	(12%)	(78%)	(6%)	(6%)	(59%)	(2%)	(10%)	(57%)	(16%)
Statistical results									
*P*1*	**0·0072**	0·7521	0·0565	**0·0062**	1·0000	1·0000	1·0000	1·0000	0·7223
*P*2*	**0·0158**	**0·0004**	**0·0170**	**0·00008**	**0·00007**	1·0000	0·1567	0·4669	0·5398
*P*3*	0·1889	**0·0081**	**0·00002**	1·0000	**0·0014**	1·0000	0·3165	0·5753	1·0000

* *P*1: was as compared between site 1 isolates to site 2 isolates.
*P*2: was between the site 1 isolates to the site 3 isolates.
*P*3: was between the site 2 isolates to site 3 isolates.
Bold type: significant differences between both.

however, they varied in molecular weight with the 31, 32 and 33 kDa protein predominating in isolates from sites 1, 3 and 2 respectively (Table 2). OspB was more prevalent among isolates from sites 1 and 3 than site 2; the 34 kDa protein predominated in site 1 and the 35 kDa protein in site 3. OspC was detected in 69. 65 and 83% of isolates from sites 1, 2 and 3 respectively with the 22 kDa protein being the most prevalent in all three sites.

The distribution of typing groups in different areas

Between the sites of isolation, the distribution of the main typing groups differed significantly (Fig. 1*b*). In site 1, group I and III were most frequent (38 and 42%) and group I was more prevalent than in site 2 (0%, $P = 0{\cdot}0072$) and in site 3 (3%, $P = 0{\cdot}0008$). Group III was the main group in site 2 (87%). Its presence differed significantly from site 1 (42%, $P = 0{\cdot}0058$) and site 3 (24%. $P = 0{\cdot}00004$). Group II was more frequent in site 3 (60%) than in site 1 (12%. $P = 0{\cdot}00007$) and site 2 (12%, $P = 0{\cdot}0014$). About 8% of isolates from each site belonged to groups IV, V, VI or VII.

The distribution of the different Osp phenotypes

Differences in the distribution of the Osp phenotypes according to the expression pattern of the different Osps were observed between the three areas studied (Fig. 2). Phenotype AC (OspA + OspC) was the most frequent in sites 1 and 2. whereas phenotype ABC (OspA + OspB + OspC) predominated in site 3 (54%) and was less frequent in site 2 (12%) than in site 3 ($P = 0{\cdot}0038$). Phenotype A (OspA only) was more prevalent in site 2 (29%) than in the other sites.

The immuno-reactivity with different MoAbs and PoAbs

About half of isolates from sites 1 and 3 reacted with MoAb H5332. The isolates

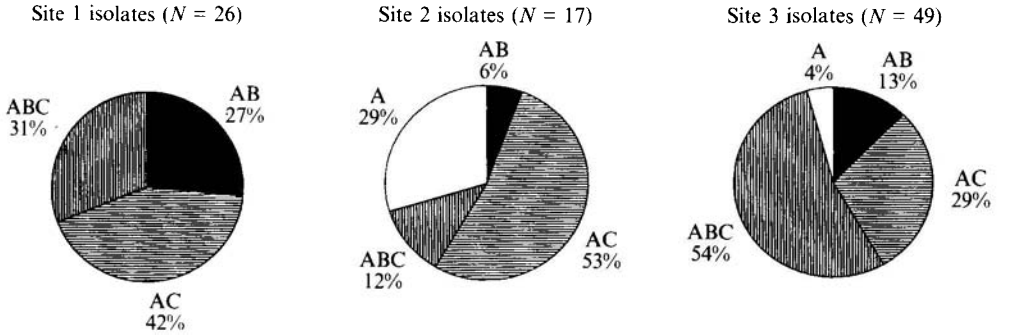

Fig. 2. Relative distribution of the Osp phenotypes (in percentage) in *B. burgdorferi* isolates derived from the three sites of isolation. A, expression of OspA only; AB, expression of OspA and OspB; AC, expression of OspA and OspC; ABC, expression of OspA, OspB and OspC.

Table 3. *The reaction of* B. burgdorferi *isolates derived from three different sites with specific mono- and polyclonal antibodies*

	MoAbs							PoAb
	H5332	LA-2	LA-4	LA-31	LA-25	LA-27	LA-7	anti-22 kDa
Site 1	13	10	3	**21**	5	**11**	4	11
(N = 26)	(50%)	(30%)	(12%)	(81%)	(19%)	(42%)	(15%)	(42%)
Site 2	5	3	0	7	2	1	0	8
(N = 17)	(29%)	(18%)	(0%)	(41%)	(13%)	(6%)	(0%)	(50%)
Site 3	29	18	9	14	6	5	3	**37**
(N = 49)	(59%)	(37%)	(18%)	(29%)	(12%)	(10%)	(6%)	(76%)
Statistical results								
*P1**	0·2194	0·1874	0·2658	0·0200	0·6897	**0·0149**	0·1498	1·0000
*P2**	0·4733	1·0000	0·5256	**0·00002**	0·4984	**0·0023**	0·2270	**0·0059**
*P3**	0·0490	0·2273	0·0977	0·3566	1·0000	1·0000	0·5692	0·0386

* *P1*: was as compared between the site 1 isolates to site 2 isolates.
P2: was between the site 1 isolates to site 3 isolates.
P3: was between the site 2 isolates to site 3 isolates.
Bold type: significant differences between both.
MoAbs: monoclonal antibodies.
PoAbs: polyclonal antibodies.

from site 1 reacted more frequently (81%) with MoAb LA-31 (anti-OspA of B31) than isolates from site 3 (29%; $P = 0{\cdot}00002$) (Table 3). Reactions of the isolates with MoAb LA-27 (anti-OspB) were more frequent in site 1 (42%) as compared to site 2 (6%) and site 3 (10%); the differences were statistically significant ($P = 0{\cdot}0149$ and $P = 0{\cdot}0023$). In contrast, 76% of site 3 isolates reacted with anti-22 kDa/NE4 (PoAb) and this was significantly different from isolates from site 1 (42%, $P = 0{\cdot}0059$).

To reveal the number B31-like strains in each area, we also compared our isolates with the *B. burgdorferi* strain B31, a prototype strain isolated from *I. dammini*. Five of the site 1 isolates had the same Osp phenotype as B31. Five additional site 1 isolates and three site 3 isolates, all of which expressed the OspC

protein, were similar to the B31 strain in the protein Mw of their OspA (31 kDa) and OspB (34 kDa). However, only two of the site 1 isolates displayed the same reactivity as B31 with the MoAbs described by Barbour and colleagues [8], Kramer and colleagues [15] and Wallich and colleagues [12].

DISCUSSION

Our study confirms the antigenic heterogeneity of the European *B. burgdorferi* isolates [8, 9, 11, 12]. In this study we compared isolates from ticks from three endemic areas and showed significant differences in the distribution of the different phenotypes among the isolates of these sites.

According to the molecular weight of OspA and OspB (Fig. 2), each endemic area presented a main typing group which differed significantly from the two other sites: group I prevailed in site 1, group III in site 2 and group II in site 3. A recent study demonstrated that the molecular weights of OspA and OspB and the representative phenotypes of borrelia isolates from patients with disseminated Lyme borreliosis were different from those obtained from patients with the cutaneous form of Lyme borreliosis [20]. It was reported that most skin isolates presented proteins of 32 kDa (OspA) and 35 kDa (OspB) (group II in our study), whereas the isolates from the disseminated Lyme borreliosis patients expressed an OspA of 32·5 kDa and an OspB of 33–34 kDa (groups III and V in our study). In view of our results, it may be suggested that the clinical manifestations of Lyme borreliosis may differ in different geographical areas. This hypothesis remains to be confirmed.

Immunoreactivity of the isolates varied between the different sites of isolation. Site 1 isolates reacted most with MoAbs LA-31 and LA-27. Site 3 isolates reacted most frequently with the MoAb H5332 and PoAb anti-22 kDa/NE4. In addition, the frequency of isolates reacting with MoAb LA-2, a MoAb which recognizes a protective epitope against *B. burgdorferi* infection [21], was different in each site. On the other hand, the comparison of our isolates with the strain B31 showed that only two isolates presented exactly the same reactivity with the MoAbs described by Barbour and colleagues [8], Kramer and colleagues [15] and Wallich and colleagues [12]. In view of this, it is suggested that European isolates could elicit specific antibody responses during infection, which differ from one site to another site and which is different from that induced by the strain B31. Therefore, a correct selection of the antigen or antigens seems to be necessary for the serodiagnosis of Lyme disease.

The different protein profile and immunoreactivity of the isolates from different geographical locations may account for seroconversion in people who do not develop Lyme borreliosis, as described in Aarberg (site 3, in this study) where 26% of an asymptomatic population was seropositive by ELISA and Western blotting [22, 23]. Among isolates from the Aarberg area (site 3), 83% expressed the 21–23 kDa proteins and 76% reacted with PoAb anti-22 kDa/NE4 and this was significantly different from the isolates from site 1. Several studies showed that these proteins are important immunogens which elicit the antibody response early after the tick bite [5, 17–19]. It remains to be elucidated whether these proteins could be responsible for the presence of asymptomatic seropositive people in

Aarberg by eliciting a protective antibody response. Another explanation could be that some of the strains present in this area were less pathogenic or non-pathogenic and that could depend on their antigenic profiles.

Heterogeneity observed among *B. burgdorferi* isolates from different sites which are fairly close to each other may have implications for human health if some strains are capable of producing early or late manifestations of Lyme borreliosis, on serodiagnosis and also on the production of protective vaccine since OspA and a 22 kDa protein are actually vaccine candidates. In fact, several studies showed that these antigens elicit protective antibody responses in animal models [21, 24–26].

The reasons leading to such a geographic diversity remain unknown but could be due to differences in the reservoir hosts in these three areas. However, our results suggest that studies on the local distribution of *B. burgdorferi* strains in each endemic area should represent an important step toward understanding the epidemiology of Lyme borreliosis, toward improving serological testing and toward developing an efficient vaccine for populations exposed to bites by infected ticks.

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